Int Arch Occup Environ Health (2009) 82:275–278
DOI 10.1007/s00420-008-0323-3

SHORT COMMUNICATION

Comments on "Radiofrequency electromagnetic fields (UMTS, 1,950 MHz) induce genotoxic effects in vitro in human fibroblasts but not in lymphocytes" by Schwarz et al. (Int Arch Occup Environ Health 2008: doi: 10.1007/s00420-008-0305-5)

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Received: 26 February 2008 / Accepted: 3 April 2008 / Published online: 24 April 2008

Abstract
Background A recent publication by Schwarz et al. describes the effects of exposure of human fibroblast and lymphocytes to radiofrequency-electromagnetic fields at frequencies used for communication with mobile phones. Even at very low specific absorption rates of 0.05 W/kg, thus well below internationally accepted exposure limits, significant effects were seen in fibroblasts whose DNA molecules were damaged as assessed by their comet tail factor (CTF) in the comet assay.
Areas of concern The CTF mean values and the standard deviations of the replicates revealed very low coefficients of variation, ranging from 1.2 to 4.9% (average 2.9%), which are in contrast to much higher variations reported by others. Moreover, inter-individual differences of the CTF values strongly disagree with the previously published data from the same group of researchers.
Conclusion The critical analysis of the data given in the figures and the tables furthermore reveal peculiar miscalculations and statistical oddities which give rise to concern about the origin of the reported data.

Keywords Electromagnetic fields · UMTS · Non-thermal · DNA · Comet assay

Introduction

The question of whether or not radiofrequency-electromagnetic fields (RF-EMF) used for mobile communication pose a health risk is being intensely discussed between politicians, health officials, physicians, scientists, and the public. Whereas the majority of scientific publications do not indicate that these non-ionizing RF-EMFs cause biological damages at levels below the thermal threshold (Sommer et al. 2007; Tillmann et al. 2007; Vijayalaxmi and Obe 2004), some investigations demonstrated such effects. When replicated, however, even those studies were found to be non reproducible. One well-known example is the study by Repacholi et al. (1997)who have reported higher incidences of lymphoma in transgenic mice which were exposed to pulsed EMF at 900 MHz (Repacholi et al. 1997). Two independent replication studies did not confirm the earlier findings (Oberto et al. 2007; Utteridge et al. 2002).

Of particular importance is the possible damage of DNA molecules by EMF exposure. Despite the fact that no biophysical mechanism has been identified for such interactions, some results of studies apparently showed DNA damages which, if such studies were found to be reproducible, would give rise to concern about immediate and long-term safety issues of mobile phone use. In 2005, it was shown by a group of researchers from the Medical University Vienna that DNA molecules of human fibroblasts and rat granulosa cells, when exposed to EMFs at 900 MHz, were significantly damaged, as shown by the comet assay (Diem et al. 2005). A replication study, using the same exposure apparatus, however, did not confirm these initial findings (Speit et al. 2007). The same group from Vienna recently published their findings on human fibroblasts and lymphocytes, this time exposing the cells to RF-EMFs at frequencies of the new mobile phone communication standard UMTS at around 1,950 MHz (Schwarz et al. 2008). Like in their earlier investigation, exposed fibroblasts' DNA molecules were found to be severely damaged, even at specific absorption rates (SAR) of 0.05 W/kg, thus far

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below the recommended exposure limits for whole-body exposure (0.08 W/kg) and partial-body exposure (2 W/kg), respectively, of the general public (ICNIRP 1998).

Areas of concern

Before the problems of the publication of Schwarz et al. are addressed, it is important to briefly summarize how the cells, after treatment (exposure, sham exposure, negative or positive control), were analyzed for their DNA damages: cells (10,000–30,000 per slide) were placed on slides in agarose and treated with lysis solution. After incubation (to allow unwinding of the DNA molecules), electrophoresis was performed so that the DNA molecules or fragments thereof moved along the slide to the anode. After electrophoresis, DNA molecules were stained with ethidium bromide, and the "comets" (tailing of the DNA spots) were inspected and examined microscopically at 400× magnification. The fraction of total DNA present in the tail of the comet reflects the frequency of DNA breaks. Per slide, 500 cells were examined. The comets were manually classified into five categories from A (no damage, no tail) to E (severe damage, longest tail). The resulting comet tail factor (CTF) was calculated per slide by multiplying the numbers of cells in each category with numbers representing the average of damage (in % tail DNA) of each category. These calibration factors, derived from previous work, are 2.5% for A cells (no tail), 12.5% for B cells, 30% for C cells, 67.5% for D cells, and 97.5% for E cells (longest tail). The cumulative sum of the products of numbers of cells × factors, divided by the number of cells (500) yielded the final result of CTF for each slide. For example, the following numbers of cells were counted: A, 445 cells; B, 39 cells; C, 13 cells; D, 2 cells; E, 1 cell. The resulting CTF value would be 4.45. These data were actually extracted from one of the data of sham-exposed cells given in Table 2 of the paper by Schwarz et al.

Low standard deviations

Per data point (i.e., for each of the five SAR values), three independent replicates with three cell culture dishes each were used for each treatment condition. It is evident that the numbers of severely damaged cells belonging to category E have a large impact on the CTF value for each slide. In the above mentioned example, one single E cell more or less would change the CTF value of the slide substantially to 4.64, or 4.26, respectively. Surprisingly, the coefficients of variation for the number of E cells of sham-exposed and negative control samples (both having the lowest numbers of E cells), as calculated by dividing the standard deviations by the respective means, is much higher (on average 57%) than the coefficients of variation for the respective CTF values (on average 4.0%). In other words, the very low coefficients of variation of the overall CTF values are difficult to explain, even provided that absolutely no biological or methodological variation would exist.

This argument is further underlined by looking at all coefficients of variation of all 20 CTF values given in Table 2 and Fig. 1 of the Schwarz et al. paper: on average, coefficients of variation are 2.9% and never exceed 5%, which is truly remarkable for this kind of biological experiment with a large number of possible confounders and methodological inaccuracies, among them differences in the cells' status and cycle, possible differences in cell culture conditions (from at least 15 independently performed experiments), differences in exposure to EMFs and UV, variations during electrophoresis and staining, and, most importantly, differences in microscopic examination and manual classification. What is even more surprising: the coefficients of variation are lower at higher CTF values: in sham-exposed cells or negative controls, the average coefficients of variation are 3.9 and 4.1%, respectively, whereas in RF-EMF exposed cells, the coefficients of variation are on average 2.6%, and in positive controls (irradiated with UV) only 1.2%. These extremely low variations are biologically and methodologically incomprehensible. For example, the SAR variations were already reported to be 26%, thus 10 times as large as the variations in the biological answer of the exposed cells. Furthermore, the low standard deviations are also in sharp contrast to results of a study (Speit et al. 2007) where the authors tried to replicate earlier results from the group of Vienna showing DNA breakage in cells exposed to 900 MHz RF-EMFs (Diem et al. 2005). Using the same cells as in the investigation by Schwarz et al., the authors found much higher coefficients of variation on the order of 30–40%. In this context a statement in the paper by Schwarz et al. is interesting: "Due to the scoring of 500 cells, being about ten times the cells usually processed by computer-aided image analysis, standard deviations become very low." Presumably, Schwarz et al. refer to the paper by Speit et al. where exactly 50 cells per slide were analyzed by means of a computer-assisted evaluation system for the DNA comets. It is, however, well known that the standard deviation does not depend on the number (n) of a sample, unlike the standard error. That in fact standard deviations were calculated in their publication is evident when looking at a publication by the same group (Rüdiger et al. 2006) where original (raw) data were presented in response to a critical letter (Vijayalaxmi et al. 2006) in reference to the two previous publications by the researchers from Vienna (Diem et al. 2005; Ivancsits et al. 2005). The standard deviations were in the same range as in the recent paper by Schwarz et al.

Unexpected low standard deviations are also seen in the time course study (Fig. 3) of the Schwarz et al. paper. Whereas after 4 h no effects by exposure are seen, the CTF values are significantly increased after 8 and 12 h of exposure with very low standard deviations. CTF values of sham-exposed and negative control cells are statistically indistinguishable and almost constant (range between 4.7 and 4.9). For these data ($n = 7$ for sham-exposed cells and $n = 7$ for negative controls), the coefficients of variation between the (independent) experiments were only 2.1 and 1.2%, respectively, thus even lower than the coefficients of variation between replicates which were reported to be 4.2% for "unexposed" samples. These low coefficients of variation are therefore statistically impossible.

The recent data by Schwarz et al. are also in sharp contrast to their own, previously published results (Diem et al. 2002), where inter-individual coefficients of variation for CTF values were reported to be on the order of 25–30% with age as a major factor. In the present paper (Schwarz et al. 2008) (Fig. 6a), inter-individual differences (coefficients of variation) for CTF values of cells from donors aged 6, 29, and 53 years, respectively, were only 6.1% (sham exposed), 3.8% (exposed), 7.1% (negative controls), and 4.0% (positive controls), respectively. Also, these low coefficients of variation are therefore difficult to comprehend.

Calculation errors and statistical analyses

The sums of the average values of all cell types (A–E) as given in Table 2 of the Schwarz et al. paper should be 500 since this was the number of cells which were analyzed. This is in fact the case for exposed and sham-exposed cells where the sums are 500 ± 0.2, the small deviations probably being due to rounding errors. In positive and negative controls, however, there are consistently different cell numbers with differences up to 14.6 cells.

The statistical analysis to check for significant effects of exposure was done by the non-parametric Mann–Whitney–Wilcoxon test, comparing $n = 3$ values of exposed cells with the combined ($n = 6$) values of sham-exposed and negative control cells. This way to analyze the data is odd, for several reasons. The data in Table 2 reveal that the variances of the CTF values of the three groups for each SAR value with $n = 3$ were statistically not different between exposed, sham-exposed and negative control cells, as tested by the F-test for equal variances. Thus, a parametric test would have been possible with much better significance levels by just comparing sham-exposed and exposed cells which should have been the difference of interest. This was actually the way in which the data from the previous study by the group were analyzed (Diem et al. 2005). In fact, based on the data given in Table 2 of the Schwarz et al. paper, all differences between sham-exposed and exposed CTF values turned out to be highly significantly different ($p < 0.001$) when using the parametric Student's t test. In none of these tests were the variances between the groups significantly different. Why the authors decided to perform a non-parametric test with a maximum level of significance of $p = 0.0238$ remains enigmatic. It is, however, interesting to note that a non-parametric test with $n = 3$ in both groups (exposed and sham-exposed) would not have been possible because irrespective of the differences, the lowest p value would be 0.1. In other words, it was essential to combine the CTF values of negative controls and sham-exposed cells to be able to perform a non-parametric test in the first place. This is only possible if the negative controls (cells which were placed in the incubator) and sham-exposed cells (which were placed in the exposure apparatus but were not exposed) showed about the same CTF values. Apparently and surprisingly, this was the case.

Summary and conclusion

The paper by Schwarz et al. (2008) apparently supports the earlier findings of the group (Diem et al. 2005), again showing significant deleterious effects of RF-EMF on DNA molecules of human fibroblasts (please note that the former name of the author Kratochvil was Diem). Despite the lack of any biophysical mechanism which would be able to explain such interactions, the results not only confirm the group's previous findings, but they apparently extend them to another frequency range (UMTS, around 1,950 MHz) and to lower SAR levels which are well below internationally accepted exposure limits for the general public (ICNIRP 1998).

The arguments given in this paper, focusing on the effects seen on DNA damage of fibroblasts, question the validity and the origin of the data published by Schwarz et al. (2008). Many of the arguments listed here, though, would be valid for the analysis of the micronuclei (MN), too (e.g., low standard deviations, low standard deviations at high MN numbers, low inter-individual differences, lack of random effects, etc.). For several reasons, the extremely low standard deviations are far too low for this kind of experiment in living cells with respect to the cells' status in many independently performed experiments, methodological variations (e.g., variations in the SAR levels), random effects of cells counted, and estimation errors due to microscopical inspection and manual classification. The statistical analysis was done inappropriately and several calculation errors are irritating. As long as no convincing evidence is provided rebutting all arguments as listed here, the paper of Schwarz et al. must be treated with extreme caution.

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